Neuronal calcium sparks and intracellular calcium ''noise''

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Intracellular calcium ions are involved in many forms of cellular function. To accommodate so many control functions, a complex spatiotemporal organization of calcium signaling has developed. In both excitable and nonexcitable cells, calcium signaling was found to fluctuate. Sudden localized increases in the intracellular calcium concentration—or calcium sparks—were found in heart, striated and smooth muscle, *Xenopus Laevis* oocytes, and HeLa and P12 cells. In the nervous system, intracellular calcium ions were found important in key processes such as transmitter release, repetitive firing, and gene expression. Hence, we examined whether calcium sparks also exist in neurons. Using confocal laser-scanning microscopy and fluorescent probes, we found that calcium sparks exist in two types of neuronal preparations: the presynaptic boutons of the lizard neuromuscular junction and rat hippocampal neurons in cell culture. Control experiments exclude the possibility that these calcium sparks originate from instrumental or biological artifacts. Calcium sparks seem to be just the tip of the iceberg of a more general phenomenon of intracellular calcium ''noise.'' We speculate that calcium sparks and calcium noise may be of key importance in calcium signaling in the nervous system.

Calcium ions have a wide variety of important functions in the activity of the nervous system. They control transmitter release, synaptic plasticity, firing rate, gene expression, and cell death (see refs. 1 and 2). There is no doubt that calcium ions entering through the surface membrane have a major function in many of these processes. In recent years, however, a growing body of evidence suggests that calcium released from intracellular stores also may play an important role in calcium control of neuronal function (3–5). Hence, the study of intracellular calcium dynamics is significant in this context. An exciting way to study such dynamics is through the examination of calcium sparks.

Calcium sparks are sudden localized increases in intracellular calcium concentration ($[Ca^{2+}]_{in}$). First described in *Xenopus laevis* oocytes and named calcium puffs (6), they were later found in heart muscle (7), skeletal muscle (8), smooth muscle (9), PC12 cells (10), and HeLa cells (11, 12). Calcium sparks seem to be of great functional significance in processes regulated by calcium in health and disease (9, 13, 14). The nomenclature (sparks or puffs), the definition, and the size of calcium sparks vary among preparations; however, most of the studies point out that they are a part of intracellular calcium dynamics. Because $[Ca^{2+}]_{in}$ is of great importance in the function of several key processes in the nervous system, we examined whether such sudden and localized increases in $[Ca^{2+}]_{in}$ also occur in neurons. We report here that calcium sparks exist in two different types of neuronal preparations: the presynaptic boutons of the lizard neuromuscular junction and rat hippocampal cells in culture. Furthermore, we found a more generalized phenomenon that we named calcium ''noise.'' We suggest that calcium sparks and noise are important in calcium signaling in neuronal cells and, hence, in the function of the nervous system. Some of the results were published in an abstract form (15, 16).

Methods

Confocal Microscopy. The scanning was done by using the confocal microscope Phoibos 2001 of Molecular Dynamics, with a ×40/1.0-numerical aperture oil-immersion objective. Preparations were scanned first with the image-scan mode once every 1.28 or 2.56 s and then with the line-scan mode once every 10 ms.

Lizard Preparation and Fluorescence Staining. All experiments were performed on the isolated ceratomandibularis nerve-muscle preparation of the lizard *Anolis carolinensis*. The preparation was stained in physiological salt solution (in mM, 157 NaCl/4 KCl/2 $CaCl_2$/2 $MgCl_2$/5 glucose/5 Hepes, pH 7.2–7.4) containing the calcium indicator. Several fluorescent probes were used, the calcium indicators Rhod-2 AM and Fluo-3 AM and the noncalcium indicator rhodamine 123. In each case, the staining conditions were different. Rhod-2 AM was used at a final concentration of 4 μM, and the preparation was stained for 60 min. Fluo-3 AM was used at a final concentration of 10 μM, and the preparation stimulated at 0.5 Hz for 60 min. When stained with 10 μM Fluo-3 AM, stimulation at 0.5 Hz for 60 min was used during incubation to achieve better staining. Rhodamine 123 was used at a final concentration of 10 μg/ml for 15 min. After staining, the preparations were washed with normal lizard ringer containing 50 μM d-tubocurarine chloride to prevent contraction of the muscle and allowed to rest for 30 min. All experiments were done at room temperature.

Hippocampal Primary Culture and Fluorescent Staining. Neonatal Sabra rats were killed by decapitation. The hippocampi were excised rapidly, and the cells were prepared as described (17) and plated on glass coverslips previously washed and covered with poly-D-lysine (molecular weight of 30,000–70,000; Sigma) supplemented with laminin (Sigma). Cells were cultured in B27 neurobasal medium (GIBCO/BRL) at 37°C in a humidified atmosphere of 5% CO_2 until used. All experiments were performed on cells 12–20 days after plating at room temperature. The cells were washed three times with freshly prepared extracellular medium (in mM, 150 NaCl/2.5 KCl/1.25 NaH_2PO_4/1 $MgCl_2$/10 Hepes/10 glucose/1 $CaCl_2$, pH 7.4) and loaded with the calcium indicator Fluo-3 AM (5 μM) in the same medium for 30 min at 37°C. After washing, the cells were kept for 15 min at 37°C to allow deesterification of the probe, washed again, and used for fluorescence measurements. When nominal calcium-free medium was used, the last wash and resuspension of the cells were performed in a medium with no $CaCl_2$, an addition of 2 mM EGTA, and an increased concentration (2 mM) of $MgCl_2$.

Results and Discussion

Calcium Fluctuations, Outliers, and Sparks. Fig. 1 illustrates the first main experimental finding. Fig. 1 (*Left*) shows the results obtained from the large presynaptic boutons of the lizard neuromuscular junction, and Fig. 1 (*Right*) shows the results from hippocampal neurons in cell culture. Fig. 1 *A1* and *B1* shows 3D reconstructions of the fluorescence calcium signals in each

Abbreviations: $[Ca^{2+}]_{in}$, intracellular calcium concentration; 3D, three-dimensional.

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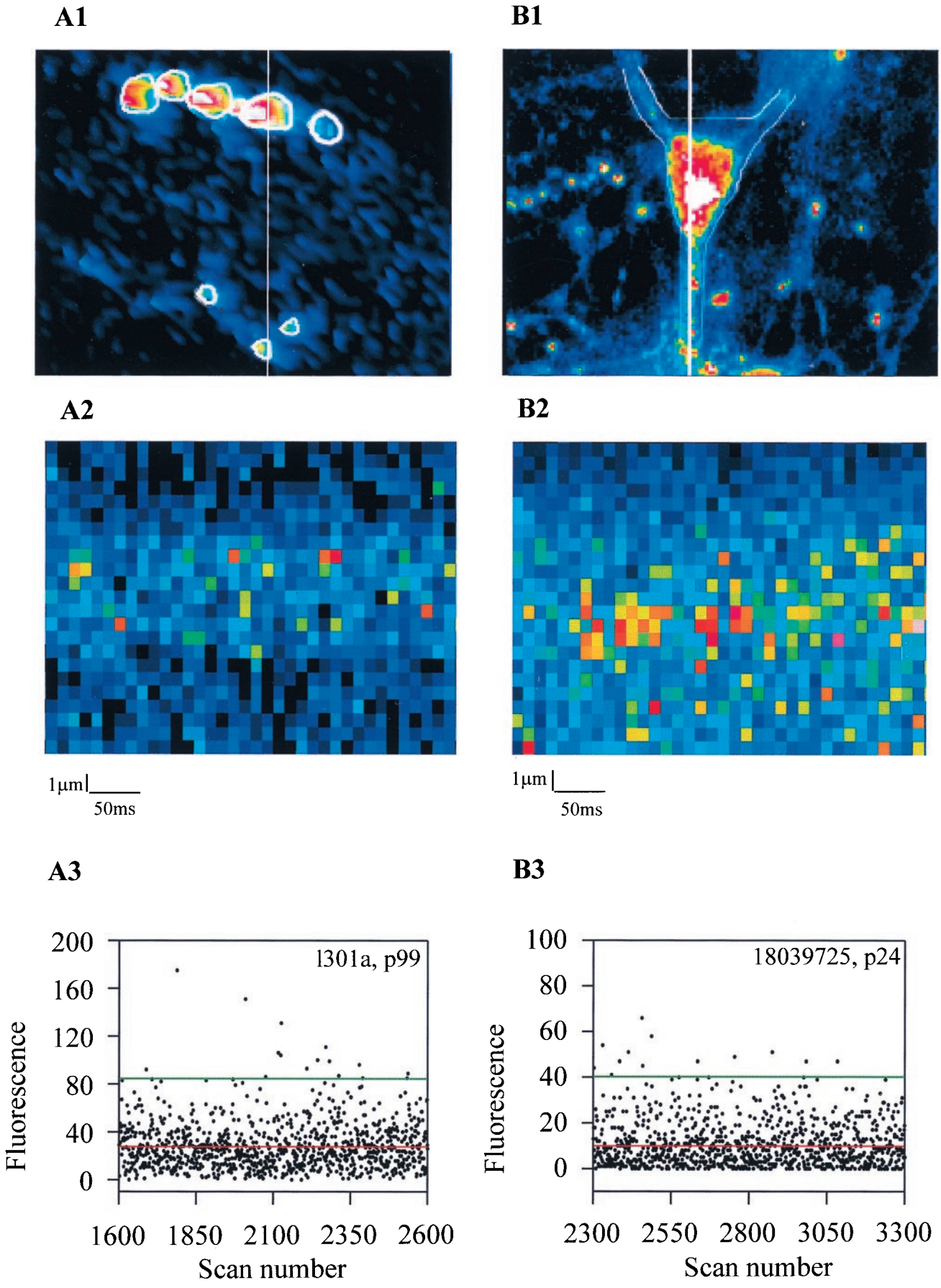

Fig. 1. (*Legend appears at the bottom of the opposite page.*)

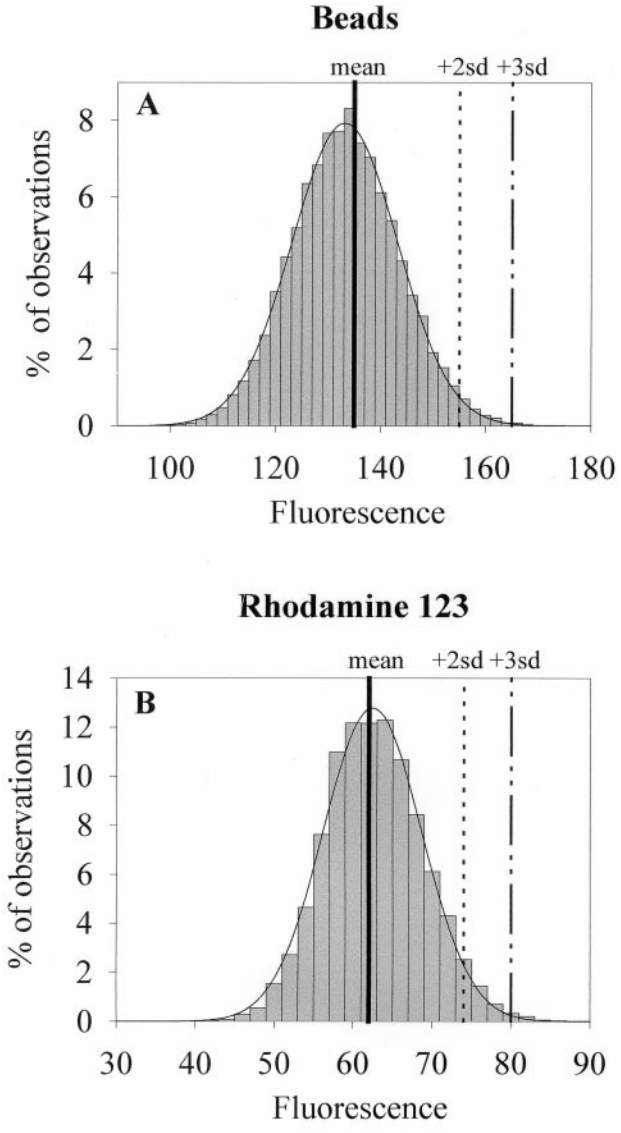

Fig. 2. Distribution of fluorescence in beads and cells loaded with a noncalcium fluorescent probe. (*A*) A histogram of the fluorescence distribution (bars) in fluorescent beads (two pixels; 19,900 scans) and its fits to a Gaussian distribution (solid line). (*B*) A histogram of 12,000 scans (bars) from four pixels in a presynaptic bouton stained with rhodamine 123, and it fits to a Gaussian distribution (solid line).

preparation, obtained by image scans in different focal planes. Because the image scans are too slow to detect calcium sparks, a line scan was used. The location of the line, scanned repeatedly every 10 ms to obtain the fast calcium signals, is shown in white.

Table 1. Fluorescence outliers

Preparation	No.	Probe	Events, %*
Beads	7	Rhodamine-like	0.15 ± 0.01
Synaptic boutons	9	Rhodamine 123	0.29 ± 0.03
Synaptic boutons	9	Rhod-2 AM	1.09 ± 0.1
Synaptic boutons	7	Fluo-3 AM	1.47 ± 0.06
Hippocampal cell, normal medium	9	Fluo-3 AM	0.9 ± 0.05
Hippocampal cell, nominally calcium-free medium	6	Fluo-3 AM	1.08 ± 0.09

*Data are shown as means ± SEM.

Usually 3,000–10,000 line scans were performed in each preparation. The intensity of the fluorescence is color coded; red is the highest fluorescence, corresponding to high $[Ca^{2+}]_{in}$. One can easily see that the calcium signal fluctuates and occasionally high calcium (red) signals appear, indicating a localized increase in $[Ca^{2+}]_{in}$. In previous work done on the heart and on skeletal muscle, these localized events were defined as calcium sparks; for oocytes and other cells, these events were named calcium puffs (6–8, 18). Fig. 1 *A3* and *B3* shows the numerical representations of the fluorescence calcium signals in selected locations in the two preparations. Such sudden increases in $[Ca^{2+}]_{in}$ were observed in all 14 boutons examined at the presynaptic terminal of the lizard and in 15 rat hippocampal neuronal preparations. Hence, it seems that calcium sparks exist also in neurons. However, before accepting that these sudden increases in $[Ca^{2+}]_{in}$ represent a definitive finding, namely the existence of calcium sparks in the nervous system, it is necessary to rule out obvious artifacts.

Fluorescence Signals from Fluorescent Beads and from Boutons Stained with Noncalcium Indicator. There are two possible sources for artifactual calcium signals in our experimental system: artifacts arising from the confocal laser-scanning microscopy system and artifacts coming from biological sources. To assess the possible contributions of these two sources, experiments were performed on artificial fluorescent beads and on preparations stained with a noncalcium indicator. The results of these two sets of control experiments are shown in Fig. 2. Fig. 2*A* shows the distribution of the fluorescence signals obtained in artificial beads. As expected, the fluorescence signals are not uniform, and they are distributed around the mean fluorescence. A Gaussian fit of the point-spread distribution of the results shows a very good fit. (For photon fluctuations, one would expect the distribution of signals to follow a Poisson distribution, but if the mean is high enough, a Gaussian distribution is indistinguishable from a Poisson distribution.) As a check, we counted the number of scans above the mean ± 3 SD. The resulting percentage of outliers was close to 0.135%, as expected from a Gaussian distribution (mean of 0.15 in seven experiments). These results show that the confocal laser-scanning system does not produce

Fig. 1. (*On the opposite page.*) Neuronal calcium sparks in presynaptic boutons at the lizard neuromuscular junction and at cultured hippocampal cells. (*A1*) A three-dimensional (3D) representation of the presynaptic terminal at the lizard neuromuscular junction. The 3D projection consists of a series of single sections obtained every 0.5 μm in the *z* plane and put together by adding their fluorescence. The vertical line passed through the terminals represents the position of the line scan. The white line surrounding the boutons delimits their perimeter. (*A2*) A small part of several plotted line scans presented in pseudocolor. The vertical line represents 1 μm (two pixels), and the horizontal line represents 50 ms. Pixels with high calcium-derived fluorescence are shown in orange or red. (*A3*) A plot showing the calcium-induced fluorescence in 1,000 scans of one pixel as a function of scan number in the hippocampal cell. The red line is the mean, and the green line is the mean + 3 SD. Similar experiments were performed in hippocampal cells in culture. (*B1*) A 3D reconstruction of a hippocampal cell 14 days after plating. The vertical white line crossing through the 3D representation indicates the position of the line scan shown below. The white line surrounding the cell delimits its perimeter. (*B2*) A small part of several line scans in the hippocampal cell pseudocolor. Pixels with high calcium-derived fluorescence are shown in orange or red. (*B3*) A plot showing the calcium-induced fluorescence in 1,000 scans of one pixel as a function of scan number in the hippocampal cell. The red line indicates the mean fluorescence, and the green line is the mean ± 3 SD. l301a and l8039725 are experiment identifiers; p99 and p24 are pixel numbers.

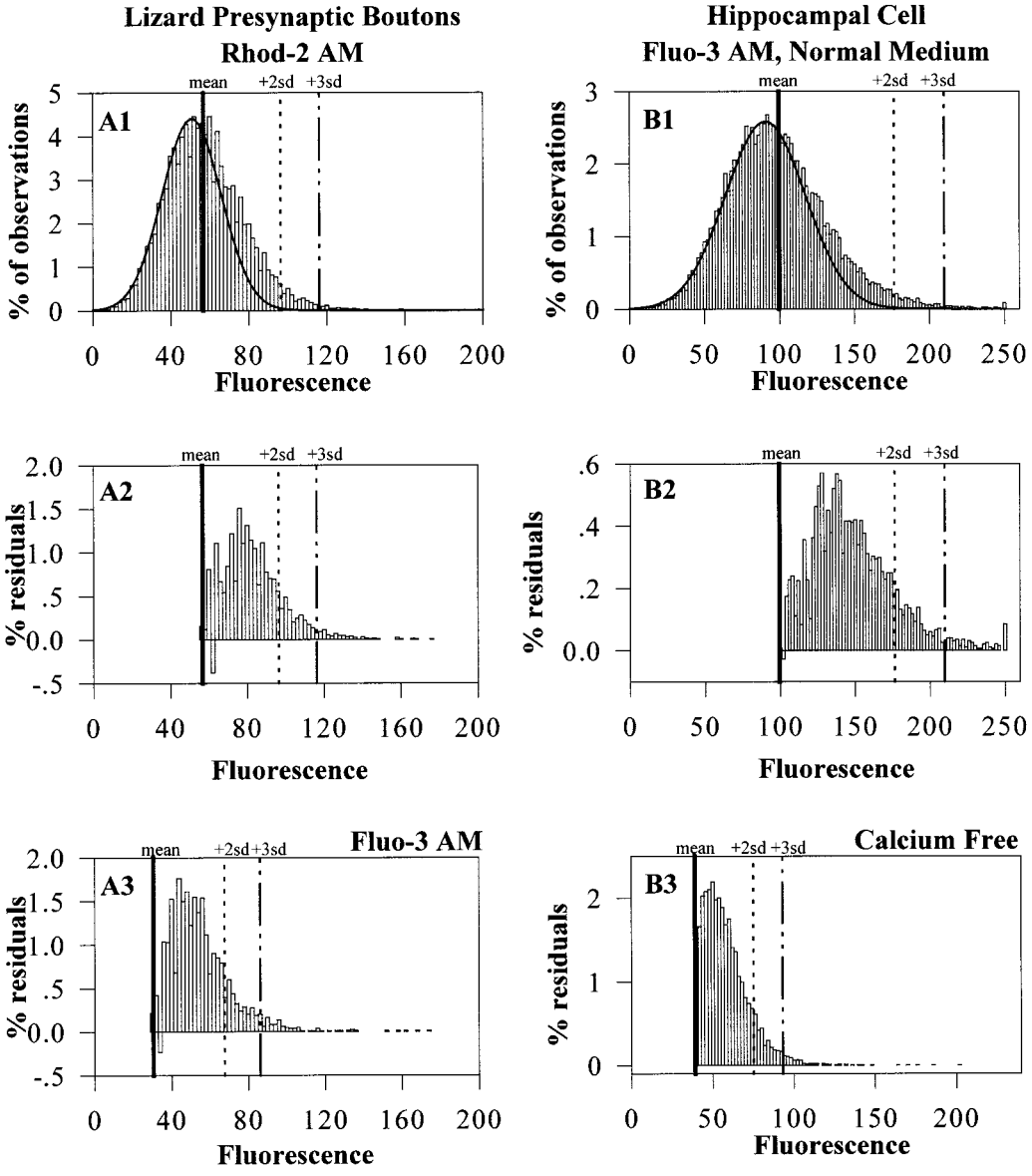

Fig. 3. (*Left*) Fluorescence distribution and residuals in presynaptic boutons. (*A1*) A histogram of 18,000 scans from six pixels in a presynaptic bouton stained with Rhod-2 AM superimposed with a Gaussian fit (solid line). (*A2*) A histogram of the residuals above the mean, calculated by PSTAT. (*A3*) A histogram, as in *A2*, for Fluo-3 AM-stained preparation. (*Right*) Fluorescence distribution and residuals in hippocampal cells. (*B1*) A histogram of the fluorescence distribution (bars) in hippocampal cells (two pixels; 19,900 scans) and its Gaussian fit (solid line). (*B2*) A histogram of the residuals' fluorescence distribution in a hippocampal cell in normal medium. (*B3*) A histogram, as in *B2*, for nominal calcium-free medium.

"spark-like" sudden increases in fluorescence. (At very low laser power, the number of outliers is larger; hence, higher laser powers were used in biological experiments.) Fig. 2*B* shows a similar analysis performed on a living tissue: presynaptic boutons of the lizard neuromuscular junction stained with rhodamine 123, a noncalcium fluorescent probe. The experimental data can easily be fitted with a Gaussian distribution, and the number of outliers was slightly above what was expected from the Gaussian distribution ($n = 9$; mean = 0.29; SD = 0.09; SEM = 0.03). For comparison, the mean percentage of events above mean ± 3 SD in preparations stained with the calcium indicator Rhod-2 AM was 1.09% in the lizard boutons ($n = 14$; SD = 0.39; SEM = 0.104) and 0.90% in hippocampal cell cultures ($n = 9$; SD = 0.17; SEM = 0.05; see Table 1). The number of outliers was about seven times larger than it was in beads and 3.76 times larger than it was in boutons stained with the noncalcium indicator. These results show that localized increases in $[Ca^{2+}]_{in}$ occur in these two preparations and that these increases do not originate from instrumental or biological artifacts.

Similar experiments were performed in lizard synaptic boutons stained with the calcium indicator Fluo-3 AM. The results with this indicator are even more striking than those obtained with Rhod-2. The mean number of outliers was 1.47% ($n = 7$; SD = 0.15; SEM = 0.056), which is about 10 times higher than it was in beads.

Calcium Noise. Having established that the confocal fluorescent signals follow a Gaussian distribution for the artificial beads and for the noncalcium indicator rhodamine 123 but have a "tail" of high fluorescent values for the calcium indicator experiments in both neuronal preparations, we attempted to determine the size of this extra calcium noise. Three different methods were employed to separate between the Gaussian components and the

extra calcium noise (residuals): a Gaussian fit of the entire distribution, a Gaussian fit of the ascending limb of the distribution, and a symmetry analysis. All three methods yielded similar results: the extra calcium noise is about 25% of the total fluorescence signal. This finding is illustrated in Fig. 3 with the ascending limb method. Fig. 3 (*Left*) shows results obtained at the synaptic boutons of lizard neuromuscular junctions, and Fig. 3 (*Right*) shows the results with rat hippocampal cells. Fig. 3 *A1* and *B1* shows the distribution of fluorescence signals and the Gaussian fit. Subtracting the Gaussian fit from the experimental results yields the residuals, depicted in Fig. 3 *A2* and *B2*. These represent the extra calcium noise in synaptic boutons and in hippocampal cells. For the synaptic boutons, this extra noise was found with two different fluorescent probes, Rhod-2 and Fluo-3 (Fig. 3*A3*). Thus, the calcium outliers and the calcium sparks seem to be a small part of a much more general phenomenon of calcium noise.

Calcium Noise in Calcium-Free Medium. For many cells, it has been suggested that the calcium sparks originate from the opening of calcium channels of intracellular origin (e.g., refs. 18–20). Different mechanisms such as spontaneous activation of the calcium channels intracellular stores (6–9), activation by the opening of closely localized L type Ca^{2+} channels (21–24), or stimulation of a second messenger pathway (12, 25) have been proposed. To check whether this hypothesis applies also to neurons, we repeated the analysis on hippocampal cells in culture in nominally calcium-free extracellular medium. If calcium sparks and calcium noise originate from the entry of calcium ions from the extracellular space, then in calcium-free medium, the distribution of fluorescence signals will be similar to that seen in control bead experiments. The results shown in Table 1 and Fig. 3*B3* establish that extracellular calcium is not required for the generation of calcium sparks and calcium noise, suggesting a predominantly intracellular calcium source.

There are several obvious questions regarding the possible role of calcium sparks and calcium noise in presynaptic function. One is whether these events are responsible for the appearance of spontaneously occurring miniature synaptic potentials. These potentials depend less on extracellular calcium than on evoked release and, in principle, could originate from the occurrence of sparks. It is clear, however, that the rate of appearance of calcium sparks is too high for the observed rate of the miniature end-plate potentials. For this hypothesis to be correct, only sparks occurring at critical locations near the release machinery would result in the release of quanta of transmitter.

In other cells, it was shown that the occurrence of a spark (or a blip) can lead to a localized coordinated increase in $[Ca^{2+}]_{in}$ (11). Such an increase could have several functional consequences at the nerve terminals. It will be of interest to see whether such increases could lead to the observed oscillations in $[Ca^{2+}]_{in}$ (26) and in the release of transmitters (27). Furthermore, it was shown recently that molecules implicated in synaptic plasticity, such as calmodulin kinase II, respond in their activity to the frequency of calcium oscillations (28). If presynaptic nerve terminals respond similarly, then calcium sparks and noise could be important components in short-term and long-term modulation of synaptic transmission.

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